Effect of macerating enzyme treatment on the polyphenol and polysaccharide composition of red wines

Marie-Agnès Ducasse, a,b,c Rose-Marie Canal-Llauberes b, Marie de Lumley c, Pascale Williams a, Jean-Marc Souquet a, Hélène Fulcrand a, Thierry Docoa, Véronique Cheyniera,*

a INRA, Joint Research Unit 1083 Sciences for Oenology, 2 Place Viala, F-34060 Montpellier, France
b NOVOZYMES France, La cité Mondiale, 23 Parvis des Chartrons, 33074 Bordeaux Cedex, France
c Laffort Oenologie, 126 quai Souys, 33100 Bordeaux, France

A R T I C L E   I N F O

Article history:
Received 14 January 2009
Received in revised form 11 March 2009
Accepted 30 April 2009

Keywords:
Wine
Enzymes
Polyphenols
Tannins
Anthocyanins
Colour
Pectic polysaccharides
RG-II

A B S T R A C T

Effect of macerating enzymes on the polyphenol and polysaccharide composition of Merlot wines after 20 months of ageing was studied over three vintages (2004, 2005, 2006). Pectinase rich enzyme preparations, by degrading grape berry cell walls, led to a modification of the molecular weight distribution of polysaccharides released into the wines. Enzyme-treated wines contained more Rhamnogalacturonan II (RG-II) and less polysaccharides rich in arabinose and galactose (PRAGs) over the three vintages. The enzyme treatment also modified wine polyphenol composition. An increase of colour intensity, of derived pigments resistant to sulphite bleaching and of proanthocyanidins (condensed tannins) was observed, modulated by vintage effect. Principal Component Analysis of all the data indicated both vintage and enzyme effects. Beside the vintage effect separating the wine samples, the impact of enzyme treatment on wines was established for each year.

1. Introduction

Pectinase rich enzyme preparations obtained from Aspergillus sp. are commonly used in red wine-making to maximise the extraction of free-run juice during maceration, to aid in clarification and filtration, and to facilitate the processes (Canal-Llaubères, 1993). Pectinases act on the pectic substances which occur as the structural polysaccharides in the middle lamella and primary grape cell wall (Pinelo, Arnous, & Meyer, 2006). The polysaccharidic fraction of wines includes polysaccharides rich in arabinose and galactose (PRAGs) such as type II arabinogalactan-proteins (AGPs) and arabinans, Rhamnogalacturonans (RG-I and RG-II) coming from the pecto-cellulosic cell walls of grape berries (Doco & Brillouet, 1993; Pellerin et al., 1996; Pellerin, Vidal, Williams, & Brillouet, 1995; Vidal, Williams, Doco, Moutounet, & Pellerin, 2003), and mannoproteins (MPs) released by the yeasts during fermentation (Llaubères, Dubourdieu, & Villettaz, 1987; Waters, Wallace, Tate, & Williams, 1993). The structure and amounts of polysaccharides released in wines depend on the wine-making process and can be modified by enzyme treatment (Ayestaran, Guadalupe, & Leon, 2004; Doco, Williams, & Cheynier, 2007; Guadalupe, Palacios, & Ayestaran, 2007). Polysaccharides are one of the main groups of macromolecules of wine. They have been thoroughly studied because of their technological and sensory properties in wines. They play a role in the colloidal stability of wines through their ability to interact and aggregate with tannins. Polysaccharides do not prevent initial tannin aggregation but they influence particle size evolution (Riou, Vernhet, Doco, & Moutounet, 2002). RGII enhances tannin particle size, suggesting co-aggregation between RGII and tannins. It has been shown to decrease astringency in wine-like model solutions (Vidal et al., 2004) and is also known to inhibit hydrogen tartrate crystallisation (Gerbaud et al., 1996). The effect of enzymes on wine polysaccharide composition has been already studied (Doco et al., 2007): an increase of RG-II and a decrease of PRAGs were observed, along with a particular modification of AGPs, with loss of their terminal arabinose residues. Addition of macerating enzymes to the mash is also commonly performed to increase the extraction of phenolic compounds, and especially anthocyanins which are the red pigments of grapes, and thus to enhance wine colour intensity. However, studies on the effect of enzymes on wine colour and anthocyanin content have led to conflicting results (Sacchi, Bisson, & Adams, 2005): some publications have shown an increase of anthocyanins (Kelebek, Canbas,
2.1. Grape materials

2. Materials and methods

itative compositions were determined by phloroglucinolysis. Resistance in red wine quality, proanthocyanidin quantitative and qualitative aspects of Merlot grown in an experimental parcel located near Bordeaux in Southern France and harvested at maturity (Table 1) over three vintages (2004, 2005 and 2006). Analyses at harvest [reducing sugars (RS) measured by a colorimetric method (OIV, 2005), total acidity (TA), weight of grape berries (W), pH, anthocyanins at pH 3.2 determined by Glories’s method (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2004) (Aph3.2), absorbance at 280 nm (DO280)] were performed by SARCO (Laffort laboratory, France).

2.2. Preparation of trials

One hundred and seventy kilograms of grapes, representative sampling of the parcel, were destemmed and crushed, and distributed into 200 l stainless steel tanks. Bisulphite (6 g/l) was first added. After homogenisation, different enzymatic preparations were added: enzyme A (Vinozym Vintage FCE, Novozymes, Denmark, a preparation of pectinases, mainly containing polygalacturonase purified from cinnamoyl esterase activity for red wine-making) was applied at 3.5 g/100 kg either alone or together with enzyme B (Vinoflow FCE, Novozymes, Denmark, a purified preparation of pectinases and beta-glucanases for red wine maturation) at 5 g/l. Abbreviations used for trials are the following: control wine (control); enzyme A (A); enzymes A and B (AB), vintages: 2004 coded 04, 2005 coded 05, 2006 coded 06.

2.3. Wine-making

After 12 h of maceration at 12 °C, all fermentations were started by implanting Excellence SP (commercial yeast, Lamothe-Abiet, Bordeaux, France) at 20 g/hl and were carried out in 200 l stainless steel tanks equipped with temperature control enabling to regulate fermentation kinetics. Alcoholic fermentation started at 15 °C, the temperature was allowed to rise during fermentation and was then maintained at around 24–25 °C. When alcoholic fermentation was finished (11 days), the free-run juice of each trial was transferred to one 200 l tank. After devatting, the young wines were kept at 20–21 °C to favour malolactic fermentation using thermoregulation. Freeze dried bacteria culture was inoculated at 1 g/l to induce malolactic fermentation. Wines were filtered after 6 months of ageing on clarifying 20 × 20 cm cellulose-Kieselguhr Seitz K200 plates prior to bottling. Wine samples were stored in the wine experimental cellar until analysis. All wines were analysed after the same period of ageing, i.e. 20 months after the end of alcoholic fermentation.

2.4. Preparation of total soluble polysaccharides of wines

The wine polysaccharides were isolated as previously described (Vidal, Williams et al., 2003). Wine (2.5 ml) was evaporated in centrifugal evaporator (EZ-2, Genevac®, Ipswich, UK). The residue was dissolved in 0.5 ml of water to obtain wine concentrated five times. Ethanol (2.66 ml, 95%) containing 0.5% HCl was added to obtain a final concentration of 80% ethanol. After one night at 4 °C, wine polysaccharides were precipitated and the supernatant was eliminated after centrifugation (10 min, 15,000 rpm). The pellet that corresponds to total wine colloids was dissolved in 1 ml of H2O (Millipore). The oligosaccharides and salts contained in the total colloids were eliminated by retention on a column (4 ml) of an

Table 1

<table>
<thead>
<tr>
<th>Vintage</th>
<th>Date of harvest</th>
<th>RS g/l</th>
<th>TA g/l (H2SO4 g/l)</th>
<th>W (200 grape berries) g</th>
<th>pH</th>
<th>A4 pH = 3.2 (mg/l)</th>
<th>DO280</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>09/29/2004</td>
<td>215</td>
<td>2.8</td>
<td>379</td>
<td>3.5</td>
<td>852</td>
<td>57</td>
</tr>
<tr>
<td>2005</td>
<td>09/19/2005</td>
<td>222</td>
<td>3.64</td>
<td>312</td>
<td>3.34</td>
<td>1017</td>
<td>65</td>
</tr>
<tr>
<td>2006</td>
<td>09/21/2006</td>
<td>232</td>
<td>3.54</td>
<td>350</td>
<td>3.47</td>
<td>963</td>
<td>62</td>
</tr>
</tbody>
</table>

1: Reducing sugars; 2: total acidity; 3: weight of 200 grape berries; 4: anthocyanins at pH = 3.2 determined by Glories method; 5: absorbance at 280 nm.
ion exchange mixed resin (Mix Bed Resin AG 501-X8, Bio Rad); wine polysaccharides, not retained, were eluted by 2.5 bed volumes of H₂O. Total soluble polysaccharides were obtained after the freeze drying of water eluted materials.

2.5. Analysis of polysaccharides

The molecular weight distribution of the wine polysaccharides was determined by high-performance size-exclusion chromatography (HPSEC) using a system equipped with a 234-Gilson sampling injector (Roissy, France), a LC-10 AS Shimadzu pump (Kyoto, Japan). HPSEC elution was performed on two serial Shodex Ohpak KB-803 and KB-805 columns (0.8 × 30 cm; Showa Denkko, Japan) connected to a ERC-7512 refractometer (Erma, Japan) at 1 ml/min flow rate in 0.1 M LiNO₃. The apparent molecular weights were deduced from the calibration curve established with a pullulan calibration kit (P-400, Mₚ = 380,000 P-200, Mₚ = 186,000; P-100, Mₚ = 100,000; P-50, Mₚ = 48,000; P-20, Mₚ = 23,700; P-10, Mₚ = 12,200; P-5, Mₚ = 5800; Showa Denkko K.K., Japan). The calibration equation was log Mₚ = 28.321–1.04 × tR (tR = column retention-time at peak maximum, and r² = 0.997). Neutral monosaccharides were released after hydrolysis of wine polysaccharides with 2 M trifluoroacetic acid (75 min at 120 °C) (Albersheim, Nevins, English, & Karr, 1967). They were then converted to the corresponding alditol acetate derivatives by reduction and acetylation and quantified by GC analysis (Harris, Henry, Blakeney, & Stone, 1984) using a fused silica DB-225 (210 °C) capillary column (30 m × 0.32 mm i.d., 0.25 μm film) with H₂ as the carrier gas on a Hewlett-Packard Model 5890 gas chromatograph. The different alditol acetates were identified on the basis of their retention time by comparison with standard monosaccharides. Neutral sugar amounts were calculated relative to the internal standard (myo-inositol). Polysaccharide composition of each wine was estimated from the concentration of individual glycosyl residues, determined by GC after hydrolysis, reduction, and acetylation, that are characteristic of the known wine polysaccharides as previously described (Vidal, Williams, O'Neill & Pellerin, 2001). The calculation of the wine polysaccharide concentrations takes account of the composition of characteristic monosaccharides as well as hydrolysis yield (Doco, Quellec, Moutounet, & Pellerin, 1999).

2.6. Sample preparation and analysis of wine polyphenols

2.6.1. Analysis of anthocyanins and tannin–anthocyanin dimer adducts

Anthocyanins and tannin–anthocyanin dimer adducts were analysed by direct injection of wines into the HPLC system. HPLC–DAD analyses were performed using a Waters 2690 system equipped with an autosampler system, a Waters 996 photodiode array detector, and a Millenium 32 chromatography manager software (Waters, Milford, MA). Separation was achieved on a reversed-phase Atlantis dC18 column (250 × 2.1 mm i.d., 5 μm packing) protected with a guard column of the same material (20 × 2.1 mm i.d., 3 μm packing) (Waters, Milford, MA). The elution conditions were as follows: 0.250 ml/min flow rate; oven temperature 30 °C; solvent A, water/formic acid (95/5 v/v); solvent B, acetonitrile/water/formic acid (80/15/5 v/v/v); elution began isocratically with 0% B during 5 min, then continued with linear gradients from 0% to 10% B in 20 min, from 10% to 20% B in 15 min, from 20% to 45% B in 15 min, from 45% to 60% B in 15 min, from 60% to 80% B in 5 min, followed by washing and re-equilibration of the column. The injection volume for all samples was 5 μl. A calibration curve was established using malvidin 3-O-glucoside from Extrasynthese (Genay, France) to quantify red pigments at 520 nm as equivalent malvidin 3-O-glucoside.

2.6.2. Analysis of tannins

Tannins were analysed by HPLC after acid-catalysed cleavage in the presence of excess phloroglucinol as previously described (Kennedy & Jones, 2001). The protocol was adapted for analysis of wine tannins as follows. Wine (4 ml) was evaporated in centrifugal evaporator (EZ-Z, Genevac, Ipswich, UK). The residue was dissolved in 4 ml of water/acetic acid (98/2 v/v) and 1 ml was applied on a C-18 solid phase extraction cartridge (Waters, Vac tC18 3cc) using the RapidTrace® SPE workstation (Caliper Life Sciences, Hopkinton, MA). Sugars and organic acids were eluted with 5 ml of water/acetic acid (98/2 v/v) and phenolic compounds recovered with 8 ml of methanol. Methyl paraben (2 g/l) was added as an internal standard. An eluent volume of 8 ml was evaporated to dryness under vacuum, redissolved in 200 μl of MeOH containing 0.2 N HCl, 50 g/l phloroglucinol, and 10 g/l ascorbic acid and heated for 20 min at 50 °C. Then, an equivalent volume of aqueous 200 mM sodium acetate was added to stop the reaction. Released terminal subunits and extension subunit–phloroglucinol adducts were analysed by HPLC using a Waters 2690 system with a reversed-phase Atlantis dC18 column (Waters, Milford, MA; 5 μm packing, 250 × 4.6 mm i.d.) protected by a guard column of the same material (20 × 4.6 mm i.d.; Waters, Milford, MA) and with a Security-guard™ cartridge C18 (Phenomenex, Torrance, CA; 4.0 × 30 mm i.d.). The mobile phase was a gradient of water/acetonitrile/formic acid (80/18/2 v/v/v; solvent B) in water/formic acid (98/2 v/v; solvent A), at a flow rate of 1 ml/min at 30 °C. Proportions of solvent B were as follows: 0–5 min with 0% B; 5–35 min, 0–10%; 35–70 min, 10–20%; 70–75 min, 20–100%; and 75–80 min, 100–0%. The injection volume for all samples was 10 μl. The proanthocyanidin units were detected by a Waters 996 photodiode array detector and quantified from peak areas at 280 nm using external calibration with known concentrations of flavan-3-ol monomers from Sigma (Saint Louis, MO) and flavan-3-ol-phloroglucinol adducts, purified in the laboratory. Each analysis was performed in triplicate. These analyses gave access to total proanthocyanidin content, their mean degree of polymerisation (mDP), and the percentage of each constitutive unit (i.e. catechin, epicatechin, epicatechin gallate (%gal), epigallocatechin (%egc). The mDP was calculated as the ratio between the summed molar concentrations of all released constitutive units and the summed molar concentrations of terminal constitutive units.

2.7. Spectrophotometric measurements

Absorbance measurements were made with a SAFAS® UV mc2 spectrophotometer (Monaco) and colour indices were deduced from these absorbance measurements as previously described (Atanasova, Fulcrand, Cheynier, & Moutounet, 2002; Glories, 1984; Somers & Evans, 1977). All the absorbance measurements were converted to a 10-mm light path cell and a dilution of 1 before calculating indices. Absorbance values at 420, 520, and 620 nm were measured, 30 min after addition of acetaldehyde, in a 1-mm light path cell. Hue (H) was calculated as A420/A520 and colour intensity corrected of bisulphite (CIcorr) as A420 + A520 + A620. Wine pigments corrected of bisulphite at wine pH (WPcorr) was defined as the absorbance at 520 nm, 30 min after addition of acetaldehyde. Colour due to derivatives resistant to sulphite bleaching was determined at 520 nm in a 1-mm light path cell, 30 min after addition of a metabisulphite solution, and sulphite bleaching resistant pigment (PRs02) was calculated. Total pigments at acidic pH (PpH<1) was determined from absorbance at 520 nm with a 10-mm light path, 4 h after a 100-fold dilution in HCl 1 M. Total polyphenol index (TPI) was determined from absorbance at 280 nm with a 10-mm light path after a 100-fold dilution.
2.8. Statistical analysis

Data are expressed as the average of three measurements. ANalysis Of VAriance (ANOVA) and Principal Component Analysis (PCA) were carried out with the software XLSTAT (Addinsoft, Paris, France). The analytical data were centred and normalised before being treated by PCA.

3. Results and discussion

3.1. Effect of enzymes on wine polysaccharide profile

The polysaccharides isolated from Merlot wines have the same molecular weight distribution as previously described in Carignan (Doco et al., 1999) and Tempranillo (Guadalupe & Ayestaran, 2007) wines (Fig. 1). A first peak eluting between 14 and 16.5 min corresponds mainly to mannoproteins (MPs) released from yeast during fermentation; a second peak eluting between 16.5 and 18 min corresponds mainly to a mixture of arabinogalactan-proteins (AGPs), arabinans, and few MPs; and a third peak eluting between 18 and 19.2 min corresponds mainly to Rhamnogalacturonan II (RG-II) with AGPs and MPs of lower molecular weight. Pectinase addition leads to a modification of the molecular weight distribution of wine polysaccharides (Fig. 1). A shift from higher to lower molecular weight polysaccharides is observed in enzyme-treated wines; the decrease in the second peak indicates that AGPs and arabinans have been partly degraded to lower molecular weight polysaccharides, while the increase of the third peak may reflect a release of both lower molecular weight polysaccharides and of RGII due to an extended cell wall degradation, as shown earlier (Doco et al., 2007). The enzyme effect on the AGP peak seems to be dependent of vintage. The decrease of the AGP peak in enzyme-treated wines was more pronounced in 2004 and 2006 (Fig. 1A and C, respectively) than in 2005 (Fig. 1B). This phenomenon may be due to a difference of maturity (Table 1) of grape berries between the three vintages.

3.2. Effect of enzymes on polysaccharide composition

Concentrations in MPs, PRAGs and RG-II were estimated from the concentration of individual glycosyl residues (mannose, arabinose, galactose, rhamnose, fucose, xylose, apiose, 2-O-methyl-fucose and 2-O-methyl-xylose) determined by GC after hydrolysis, reduction and acetylation. All the mannose is attributed to yeast MPs. PRAGs, representing mainly arabinogalactan-proteins and arabinans in wines, are estimated from the sum of galactose and arabinose residues. RG-II is calculated from the concentration of apiose, 2-O-methyl-fucose and 2-O-methyl-xylose. Fig. 2 presents the concentrations of MPs, PRAGs and RG-II in mg/l in wines. The data confirms the interpretation of molecular weight distribution profiles. The use of pectolytic enzymes modified the composition of the polysaccharides released from cell walls in wines as shown by a decrease of PRAG concentration and an increase of RG-II concentration. The loss of PRAGs was more pronounced in 2006 than in 2004 (Fig. 2A and C, respectively) and was not observed in 2005 (Fig. 2B). Again, this may be related to differences in grape maturity between the three vintages, as suggested above. As expected, no significant effect of pectolytic enzymes on MPs was observed.

3.3. Effect of enzymes on wine colour

Total polyphenol and wine colour indices (Table 2) were deduced from the absorbance measurements (Glories, 1984; Somers & Evans, 1977). Colour intensity was measured after addition of acetaldehyde to release anthocyanins eventually involved in bisulphite adducts (Atanasova et al., 2002). Large vintage differences were observed in the absorbance values. Thus, the total polyphenol and colour intensity values were higher in 2005 than in 2004 and 2006 at 20 months of ageing, suggesting that higher maturity was reached in this vintage. Indeed, monitoring of colour intensity and total polyphenol index during alcoholic fermentation (Fig. 3) shows a faster increase of these indices during the first days of fermentation in 2005 (Fig. 3B) than in 2004 and 2006 (Fig. 3A and C, respectively). In 2005, the extraction kinetics were identical in enzyme-treated wines and in the control wine. In contrast, in 2004 and 2006, TPI values were significantly higher in the treated wines than in the control throughout fermentation. The total polyphenol index values after 20 months of ageing were also significantly higher in the enzyme-treated wines than in the control except for enzyme A in 2006. This suggests that the plant cell wall degradation induced by the enzyme treatment resulted in increased phenolic extraction from grapes and/or that changes in the wine polysaccharide composition enhanced the solubility and stability of phenolic compounds during wine ageing. The absorbance at 520 nm (WPcorr) and colour intensity (Ccorr) values were also higher in the enzyme-treated wines, after 20 months of ageing,

![Fig. 1. Molecular weight distribution of polysaccharides isolated from 2004 wines (A), 2005 wines (B), 2006 wines (C).](image-url)
of sulphite bleaching resistant pigments (PRSO2) in the treated treatments. The latter hypothesis is confirmed by a higher level of anthocyanin adducts in wines obtained with enzymatic treatments from the grape berries and/or higher conversion of anthocyanins for vintages 2004 and 2006, suggesting higher extraction of pigments than in the control wines over the three vintages. Tannin composition was also qualitatively different in the treated wines. Their mDP were slightly higher than in the control wines. This may be due to easier extraction of higher molecular weight tannins as a result of the increased degradation of grape cell walls induced by enzyme addition. In all cases, tannins released into the wines are mainly tannins from skins as evidenced by the %egc. A slight but significant effect of enzymes on %egc was observed in 2004 and 2006. Inconsistent effects of enzyme treatments on the concentrations of anthocyanins and tannin–anthocyanin adducts measured by HPLC were observed (Table 3 ACN, TA). It should be emphasised that the concentration of native anthocyanins reflects both their extraction from the grape and their subsequent reactions in wine. The colour properties are not related to anthocyanin concentrations but depend, on one hand, on the nature and proportions of genuine anthocyanins and derivatives formed from them during wine-making, on the other hand on copigmentation phenomenon. Anthocyanin derivatives include TA adducts which are mostly colourless at wine pH but also other types of pigments that contribute colour in wine. The relative amounts of these various compounds depend on the concentrations of tannins and anthocyanins and on their molar ratio which are modified by enzyme treatment. The increase of colour intensity of enzyme-treated wines was not

for vintages 2004 and 2006, suggesting higher extraction of pigments from the grape berries and/or higher conversion of anthocyanins to derived pigments in wines obtained with enzymatic treatments. The latter hypothesis is confirmed by a higher level of sulphite bleaching resistant pigments (PRSO2) in the treated wines than in the control wines, as genuine grape anthocyanins are bleached by sulphites while some derived pigments are not. This was not observed for the 2005 wines which showed higher values compared to the others. It seems that enzyme addition has no effect on Total Polyphenol Index (TPI) and Colour Intensity (CI) when using grapes with high initial values of these parameters, as above mentioned and shown by their monitoring of during alcoholic fermentation (Fig. 3B). Finally, the total pigment values measured in acidic conditions (ensuring conversion of all anthocyanins and derivatives to their flavylium pigment forms) were constant over the three vintages and little affected by the enzyme treatments. This indicates that the higher colour intensities measured in the 2005 wines are due to enhanced stabilisation of the pigments in the wines through formation of derived pigments or copigmentation rather than to a higher concentration of grape anthocyanins. Indeed, the sulphite bleaching resistant pigments contributed about 50% of the red colour in the 2005 wines and only 40% in the other two vintages, confirming that conversion of anthocyanin pigments occurred faster in the 2005 wines. The hue values were higher in the 2006 wines than in the other two series but were not modified by enzyme treatment.

### 3.4. Effect of enzymes on polyphenol composition

Tannin concentration (T) their qualitative composition (mDP, %gal, %egc), anthocyanin (ACN) concentration and tannin–anthocyanin adduct (TA) concentration are presented in Table 3. The enzyme-treated wines contained larger amounts of tannins than the control wines over the three vintages. Tannin composition was also qualitatively different in the treated wines. Their mDP were slightly higher than in the control wines. This may be due to easier extraction of higher molecular weight tannins as a result of the increased degradation of grape cell walls induced by enzyme addition. In all cases, tannins released into the wines are mainly tannins from skins as evidenced by the %egc. A slight but significant effect of enzymes on %egc was observed in 2004 and 2006. Inconsistent effects of enzyme treatments on the concentrations of anthocyanins and tannin–anthocyanin adducts measured by HPLC were observed (Table 3 ACN, TA). It should be emphasised that the concentration of native anthocyanins reflects both their extraction from the grape and their subsequent reactions in wine. The colour properties are not related to anthocyanin concentrations but depend, on one hand, on the nature and proportions of genuine anthocyanins and derivatives formed from them during wine-making, on the other hand on copigmentation phenomenon. Anthocyanin derivatives include TA adducts which are mostly colourless at wine pH but also other types of pigments that contribute colour in wine. The relative amounts of these various compounds depend on the concentrations of tannins and anthocyanins and on their molar ratio which are modified by enzyme treatment. The increase of colour intensity of enzyme-treated wines was not

<table>
<thead>
<tr>
<th>Trials</th>
<th>Ccorr1,2</th>
<th>H1,3</th>
<th>TPI1,4</th>
<th>WPcorr1,5</th>
<th>PR201,6</th>
<th>PmDP1,7</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004 vintage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control04</td>
<td>10.07 a</td>
<td>0.574 b</td>
<td>36.9 a</td>
<td>5.78 a</td>
<td>2.31 a</td>
<td>12.4 ab</td>
</tr>
<tr>
<td>A04</td>
<td>10.86 b</td>
<td>0.567 a</td>
<td>44.7 c</td>
<td>6.27 b</td>
<td>2.58 b</td>
<td>13.0 b</td>
</tr>
<tr>
<td>AB04</td>
<td>11.40 c</td>
<td>0.572 b</td>
<td>40.3 b</td>
<td>6.49 c</td>
<td>2.70 c</td>
<td>11.7 a</td>
</tr>
<tr>
<td>p-Value</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>2005 vintage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control05</td>
<td>14.26 b</td>
<td>0.588 b</td>
<td>41.8 b</td>
<td>8.01 b</td>
<td>3.80 b</td>
<td>13.5 b</td>
</tr>
<tr>
<td>A05</td>
<td>13.51 a</td>
<td>0.579 a</td>
<td>44.3 c</td>
<td>7.64 a</td>
<td>3.75 a</td>
<td>13.6 b</td>
</tr>
<tr>
<td>AB05</td>
<td>14.26 b</td>
<td>0.588 b</td>
<td>41.8 b</td>
<td>8.01 b</td>
<td>3.80 b</td>
<td>13.5 b</td>
</tr>
<tr>
<td>p-Value</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>2006 vintage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control06</td>
<td>9.62 a</td>
<td>0.636 b</td>
<td>36.7 a</td>
<td>5.49 a</td>
<td>2.02 a</td>
<td>12.8 b</td>
</tr>
<tr>
<td>A06</td>
<td>10.80 c</td>
<td>0.613 a</td>
<td>36.6 a</td>
<td>5.97 c</td>
<td>2.41 c</td>
<td>12.1 a</td>
</tr>
<tr>
<td>AB05</td>
<td>10.32 b</td>
<td>0.647 c</td>
<td>39.1 b</td>
<td>5.86 b</td>
<td>2.28 b</td>
<td>13.1 b</td>
</tr>
<tr>
<td>p-Value</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

1: Average of three measurements, one-factor ANOVA a same letter means no significant difference within each vintage, 2: colour intensity corrected (Abs 420 nm + Abs 520 nm + Abs 620 nm with acetaldehyde), 3: hue (Abs 420 nm/Abs 620 nm with acetaldehyde), 4: Total Polyphenols Index: absorbance at 280 nm, 5: wine pigments corrected (Abs 520 nm with acetaldehyde), 6: pigments resisting to sulphite bleaching (Abs 520 nm with SO2), 7: pigments at acidic pH < 1 (Abs 520 nm in HCl).
related to a higher concentration of residual native anthocyanins in these wines. Thus, it is presumably due to other phenomena such as increased formation of derived pigments and enhanced copigmentation as a result of increased extraction of tannins (Fulcrand et al., 2004; Salas et al., 2003). It has been shown that the colour stability of malvidin 3-glucoside solutions was decreased in the presence of flavan-3-ol monomers but enhanced in the presence of procyanidin oligomers, especially as their degree of polymerisation increased (Malien-Aubert, Dangles, & Amiot, 2002).

3.5. Vintage effect versus enzyme effect

Principal Component Analysis was performed on all the data generated from polysaccharide and polyphenol analysis and spectrophotometric measurements of the nine wines. The first and second Principal Components explained together 65.71% (PC1 36.43% and PC2 29.28%) of the total variance (Fig. 4). Projection of the wines on the first two PCs (Fig. 4A) shows a separation of the three vintages along the first two axes while control wines and enzyme-treated wines are separated along the second axis. PC1 is mainly associated positively with anthocyanins (ACN), hue (H), and tannins, and negatively with MPs, PRAGs, CIcorr, WPcorr, TPI, and PRSO2 (Fig. 4B). It contrasts 2006 wines, which show higher levels of free anthocyanins, H, and tannins, lower values of polysaccharides, and lower total polyphenol index, with the 2005 and, to a lesser extent, 2004 wines that exhibit higher values for total polyphenol and colour indices. Anthocyanin concentration is anti-correlated with CIcorr ($R = -0.76$), absorbance at 520 nm at wine pH

![Fig. 3. Evolution of colour intensity (to the left) and total polyphenol index (to the right) during alcoholic fermentation of 2004 wines (A), 2005 wines (B) and 2006 wines (C).](image-url)
WPcorr; \( R = -0.74 \) and PRSO2 \( R = -0.78 \), these three values being highly correlated, again meaning that wine colour after 20 months of ageing is mostly due to derived pigments (Fulcrand et al., 2004; Salas et al., 2004). Total polyphenol index appears correlated with colour indices but not with anthocyanins or tannins and thus is mostly contributed by derived pigments. PC2 is associated positively with mDP, \( P_{\text{mDP}} \), T–A, \%egc, and, to a lesser extent, to tannins and colour indices and negatively with PRAGs and MPs concentrations (Fig. 4B). TA and \( P_{\text{mDP}} \) are positively correlated \( (R = +0.72) \), suggesting that the T–A dimers can serve as markers of a large class of T–A adducts which represent the majority of pigments in wine acidified to pH < 1. These adducts, like anthocyanins, are coloured at acidic pH but do not contribute much to red colour at the wine pH. Within each vintage, the enzyme-treated wines are distinguished from the control wines along the second axis, on one hand, by their higher contents of RGII and lower levels of PRAGs, related to enzymatic degradation of plant cell walls, on the other hand, by their higher tannin content, higher values of mDP, indicating an increased extraction of tannins as a result of increased cell wall degradation. The 2004 wines are also distinguished from the other two vintages by their higher amounts of PRAGs and MPs.

In conclusion, the effect of enzyme treatment has been demonstrated for each vintage, especially with regards to RG-II and tannin concentrations. This effect is more or less marked depending on the vintage. In particular, the increase in colour and degradation of PRAGs was not observed in 2005, possibly due to differences in grape maturity. This should be investigated further. No effect on wine anthocyanin concentration was observed. However, in 2004 and 2006, higher colour indices in the enzyme-treated wines indicate that the enzyme treatment either resulted in higher anthocyanin extraction followed by their conversion to other pigments or favoured reaction mechanisms yielding derivatives that are pigmented in wine. Indeed, the enzyme treatment leads to an increase of pigments resistant to sulphite bleaching which contribute to wine colour. No effect was observed on the concentration of TA adducts which are mostly colourless at wine pH but appeared

### Table 3
Polyphehol composition of Merlot wines over the three vintages.

<table>
<thead>
<tr>
<th>Trials</th>
<th>( \tau_{1.2} ) (mg/l)</th>
<th>mDP(^{1.3} )</th>
<th>%gal(^{1.4} )</th>
<th>%egc(^{1.5} )</th>
<th>ACN(^{1.6} ) (mg/l)</th>
<th>TA(^{1.7} ) (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004 vintage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control04</td>
<td>425 a</td>
<td>4.65 a</td>
<td>2.38 a</td>
<td>17.90 a</td>
<td>63.3 b</td>
<td>1.35 a</td>
</tr>
<tr>
<td>A04</td>
<td>518 b</td>
<td>4.98 b</td>
<td>3.29 b</td>
<td>18.42 a</td>
<td>55.5 a</td>
<td>1.33 a</td>
</tr>
<tr>
<td>AB04</td>
<td>524 b</td>
<td>5.12 b</td>
<td>3.33 b</td>
<td>18.78 a</td>
<td>55.4 a</td>
<td>1.42 a</td>
</tr>
<tr>
<td>p-Value</td>
<td>( p &lt; 0.01 )</td>
<td>( p &lt; 0.01 )</td>
<td>( p &lt; 0.01 )</td>
<td>( p &lt; 0.01 )</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.05 )</td>
</tr>
<tr>
<td>2005 vintage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control05</td>
<td>447 a</td>
<td>4.84 a</td>
<td>2.42 b</td>
<td>22.53 a</td>
<td>25.1 a</td>
<td>1.18 a</td>
</tr>
<tr>
<td>A05</td>
<td>558 b</td>
<td>5.32 b</td>
<td>3.16 c</td>
<td>22.31 a</td>
<td>52.9 b</td>
<td>2.25 b</td>
</tr>
<tr>
<td>AB05</td>
<td>520 ab</td>
<td>6.11 c</td>
<td>1.35 a</td>
<td>23.43 a</td>
<td>58.4 c</td>
<td>1.89 b</td>
</tr>
<tr>
<td>p-Value</td>
<td>( p &lt; 0.05 )</td>
<td>( p &lt; 0.01 )</td>
<td>( p &lt; 0.001 )</td>
<td>( p &lt; 0.0001 )</td>
<td>( p &lt; 0.001 )</td>
<td>( p &lt; 0.01 )</td>
</tr>
<tr>
<td>2006 vintage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control06</td>
<td>572 a</td>
<td>4.96 a</td>
<td>1.68 a</td>
<td>24.35 c</td>
<td>85.2 b</td>
<td>1.62 c</td>
</tr>
<tr>
<td>A06</td>
<td>583 a</td>
<td>5.18 b</td>
<td>2.10 b</td>
<td>24.02 b</td>
<td>57.6 a</td>
<td>1.31 a</td>
</tr>
<tr>
<td>AB06</td>
<td>648 b</td>
<td>5.11 b</td>
<td>2.18 c</td>
<td>23.39 a</td>
<td>83.3 b</td>
<td>1.46 b</td>
</tr>
<tr>
<td>p-Value</td>
<td>( p &lt; 0.0001 )</td>
<td>( p &lt; 0.01 )</td>
<td>( p &lt; 0.0001 )</td>
<td>( p &lt; 0.0001 )</td>
<td>( p &lt; 0.001 )</td>
<td>( p &lt; 0.01 )</td>
</tr>
</tbody>
</table>

1: Average of three measurements, one-factor ANOVA a same letter means no significant difference within each vintage, n.s. indicate no significant, 2: tannin concentration, 3: mean degree of depolymerization, 4: % of epicatechin gallate subunits, 5: % of epigallocatechin subunits, 6: anthocyanin concentration, 7: flavanol anthocyanin dimer concentration.

Fig. 4. Principal Component Analysis of the polysaccharide, polyphenol and spectrophotometric data of 2004, 2005, 2006 wines: projection of the wines on principal component 1 (PC1) and principal component 2 (PC2), control wine coded control; enzyme A coded A; enzymes A and B coded AB over three vintages: 2004 coded 04, 2005 coded 05, 2006 coded 06; (B) Correlation scatterplot of the chemical and spectrophotometric variables with PC1 and PC2.
correlated to the red colour measured under acidic conditions (pH < 1). Finally, changes in polysaccharide composition induced by enzyme treatments may impact the colloidal structure and the mouth-feel properties of phenolic compounds in wines, as shown earlier in model solutions.

Acknowledgments

We thank Frederic Veran (UMR SPO) for technical assistance and all the staff of the wine experimental cellar for assistance with the wine-making experiments.

References


